II. REMARKS

A. Status of the Claims

Claims 15, 17, 19, 24, 38, 40, and 54-62 were pending in the application at the time of the Office Action, with claims 4-11, 16, 18, 20-22, 26-37, 41-48, 52, and 53 having been withdrawn from consideration as being directed to a non-elected invention. Claims 1-14, 23, 25, 39, 49-51, and 54 have been canceled without prejudice or disclaimer. Claims 15, 17, 19, and 38 have been amended in the Amendment set forth herein. New claims 63 and 64 have been added. Support for the amendments to the claims and the new claims can be found generally throughout the specification, such as in the claims as originally filed and in paragraph [0045] (claims 63 and 64). Thus, claims 15, 17, 19, 24, 38, 40, and 55-64 are currently under consideration.

B. The Claim Objection Is Moot

Claim 54 is objected to for depending from a non-elected invention (claim 21) and is considered only with regarding to the limitations of the elected invention. Applicant notes that claim 54 has been canceled without prejudice or disclaimer. Therefore, the objection is moot.

C. The Rejections Under 35 U.S.C. §112, First Paragraph, Are Overcome

Claims 17, 24, and 57-58 are rejected under 35 U.S.C. §112, first paragraph, because the specification, while being enabling for methods wherein the transgene is expressed by the BMS cells, is said to not reasonably provide enablement for the absence of expression. Applicant respectfully traverses.

Without conceding that claims 17, 24, and 57-58 are not enabled by the instant specification, Applicant notes that claim 17 has been amended to recite "wherein said delivered cells express said transgene." Claim 24 depends from claim 17 and 19, both of which recite this limitation. Claims 57-58 depend from claim 17. Therefore, this rejection has been overcome.

D. The Rejections Under 35 U.S.C. §103 Are Overcome

1. Rejections Based on Kornowski and Hu

Claims 15, 17, 19, 24, 25, 38, 40, 54, 55, 57, 59, and 61 are rejected under 35 U.S.C. §103(a) as being unpatentable over U.S. Patent 7,097,832 ("Kornowski") and U.S. Patent 7,186,688 ("Hu"). The Examiner argues that at the time of the invention, it would have been obvious to modify the methods of Kornowski with the VEGF transgene of Hu to lead to the claimed invention. Applicant respectfully traverses.

a. No Prima facie Case of Obviousness

In rejecting claims under 35 U.S.C. §103, the Examiner bears the initial burden of presenting a *prima facie* case of obviousness. See *In re Rijckaert*, 9 F.3d 1531, 1532, 28 USPQ2d 1955, 1956 (Fed. Cir. 1993). In *KSR Int'l Co. v. Teleflex, Inc., No.* 04-1350 (U.S., Apr. 30, 2007), the Supreme Court noted that the analysis supporting a rejection under 35 U.S.C. §103(a) should be made explicit, and that it is "important to identify a reason that would have prompted a person of ordinary skill in the relevant field to combine the [prior art] elements" in the manner claimed. *KSR*, slip op. at 14. More particularly, the Court noted that "[o]ften, it will be necessary … to look to interrelated teachings of multiple patents; the effects of demands known to the design community or present in the marketplace; and the background knowledge possessed by a person having ordinary skill in the art, all in order to determine whether there was an ***apparent reason*** to combine the known elements in the fashion claimed by the patent at issue," and that "[t]o facilitate review, this analysis ***should be made explicit***." *Id.*, (emphasis added).

In the instant case, there is no *prima facie* case of obviousness because the Examiner has not set forth any explicit analysis as to why one of ordinary skill in the art would substitute a transgene set forth in Kornowski with a gene expressing a VEGF. As admitted by the Examiner,

Kornowski does not disclose intramyocardial delivery of human bone marrow stromal cells modified *ex vivo* to express a VEGF. Further, the only data set forth in Kornowski pertains to the administration of cells into "the ischemic zone" and not into normal tissue adjacent to ischemic tissue in an ischemic or diseased heart. See col. 9, lines 57-61. While Hu makes reference to VEGF generally, it does not teach or suggest administration of cells expressing a transgene encoding VEGF into normal myocardial tissue. Thus, at most one of ordinary skill in the art, when presented with Hu, would at most be motivated to deliver cells that include a transgene expressing a VEGF into ***ischemic myocardial tissue*** based on the teachings of Kornowski and not into ***normal myocardial tissue*** adjacent to ischemic tissue.

The following references demonstrate that at the time of our invention, it was widely believed that you needed to deliver the therapies to the ischemic regions of the heart, and not into non-ischemic tissue. See, *e.g.*, Patterson and Runge, Circulation 1999:2614-2616 (Exhibit 1 – see particularly page 2614) and Mack *et al.*, J. Thorac Cardiovasc Surg 1998; 115:168-77 (Exhibit 2 – see, e.g., abstract).

Further, there is additionally no *prima facie* case of obviousness because neither Kornowski or Hu appear to teach or suggest use of any of the delivery devices set forth in claims 15, 17, 19, or 38.

b. Surprising and Unexpectedly Superior Results

Even if the Examiner has set forth a *prima facie* case of obviousness, which Applicant vigorously asserts is not the case, such *prima facie* case of obviousness would be successfully rebutted by Applicant's evidence of surprising and unexpected superior results.

The Examiner is directed to Examples 1 and 2 of the present specification (paras [0048] – [0061]. Chronic myocardial ischemia was induced in juvenile cross-bred pigs. Para [0048]. After performance of baseline measurements of cardiac function, the animals were divided into

four groups: (1) Ad-$VEGF_{165}$ injection into the ischemic zone; (2) Ad-$VEGF_{165}$ injection into the normal zone; (3) Ad-βGal injection into the ischemic zone; or (4) phosphate buffered saline into the ischemic zone. Para [0052]. The blood flow data indicate that when injections are targeted to the ischmic zone, modest improvements in perfusion occur at rest. Para [0053]. However, when injections are made in to the normal zone of the myocardium, significant improvements are obswerved in blood perfusion at both rest and stress. *Id.* Results are schematically depicted in FIGS. 3-5. Furthermore, transmural blood flow reaches a much higher level of 0.815 (normal zone injections) versus 0.351 (ischemic zone injections) under stress. *Id.* and FIG. 6.

In an additional study of chronic myocardial ischemia in juvenile pigs, animals that received injections of Ad- βGal into the ischemic zone did not show significant improvement in blood flow at rest. Para [0054]-[0061] and FIGS. 6-8. A stiletto (needle injection) catheter was used for injections. [0056]. Animals that received injections of Ad- $VEGF_{165}$ into the normal zone showed trends toward improvement both at rest and with pacing. Para [0059]. Further, animals that received injections of Ad- $VEGF_{165}$ in the normal zone had higher capillary density than animals that received injections of Ad- βGal in the ischemic zone, and animals that received injections of Ad- $VEGF_{165}$ in both normal and ischemic zones had capillary density similar to those that received injections of Ad- βGal in the ischemic zone. Para [0061] and FIG. 9.

These results demonstrate that the methods of the present invention, which involve injection into normal myocardium, are surprisingly and unexpectedly superior to the prior art method, which teaches injection into ischemic myocardium.

In view of the foregoing, there is no *prima facie* case of obviousness. Therefore, it is respectfully requested that the rejection of claims 15, 17, 19, 24, 25, 38, 40, 54, 55, 57, 59, and

61 as being unpatentable under 35 U.S.C. §103(a) based on Kornowski and Hu should be withdrawn.

2. Rejections Based on Kornowski and Safi

Claims 15, 17, 19, 24, 25, 38, 40, 54, 55, 57, 59, and 61 are rejected under 35 U.S.C. §103(a) as being unpatentable over Kornowski and Safi *et al.* (Microvascular Research, 58:238-49, 1999; hereinafter "Safi"). The Examiner argues that it would have been obvious to one of ordinary skill in the art to modify the methods of Kornowski with the FGF transgene of Safi to lead to the claimed invention. Applicant respectfully traverses.

In the instant case, there is no *prima facie* case of obviousness because the Examiner has not set forth any reasonable basis for one of ordinary skill in the art to substitute any transgene set forth in Kornowski with a transgene encoding FGF-1. As admitted by the Examiner, Kornowski does not disclose intramyocardial delivery of human bone marrow stromal cells modified *ex vivo* to express a FGF transgene. Further, the only data set forth in Kornowski pertains to the administration of cells into "the ischemic zone" and not into normal tissue adjacent to ischemic tissue in an ischemic or diseased heart. See col. 9, lines 57-61. Safi does not teach or suggest administration of cells expressing a transgene encoding FGF into normal myocardial tissue adjacent to ischemic tissue in an ischemic or diseased heart of a subject. Rather, the injections is Safi were made into healthy heart tissue where there was no prior disease.

Further, there is additionally no *prima facie* case of obviousness because neither Kornowski or Safi teach or suggest use of any of the delivery devices set forth in claims 15, 17, 19, or 38.

Even if the Examiner has set forth a *prima facie* case of obviousness, which Applicant vigorously asserts is not the case, such *prima facie* case of obviousness would be successfully

rebutted by Applicant's evidence of surprising and unexpected superior results as discussed above.

In view of the foregoing, there is no *prima facie* case of obviousness. Therefore, it is respectfully requested that the rejection of claims 15, 17, 19, 24, 25, 38, 40, 54, 55, 57, 59, and 61 as being unpatentable under 35 U.S.C. §103(a) based on Kornowski and Safi should be withdrawn.

3. Rejections Based on Kornowski and Hu and Further in View of Waller

Claims 15, 17, 19, 38, 24, 54, 56, 58, 60, and 62 are rejected under 35 U.S.C. §103(a) as being unpatentable over Kornowski and U.S. Patent 7,186,688 (hereinafter "Hu"), and further in view of U.S. Patent 5,800,539 (hereinafter "Waller"). The Examiner argues that at the time of the invention it would have been obvious to modify Kornowski or Kornowski/Saffi with the allogeneic BMS of Waller to lead to the claimed invention. Applicant respectfully traverses.

For the reasons discussed above, Kornowski in view of Hu fails to render the claimed invention obvious. The discussion above is herein incorporated into this section of the response. The Examiner's citation to Waller fails to remedy the deficiencies of Kornowski and Hu because it is cited only for teaching use of allogeneic BMS cells for transplantation. Waller does not appear to teach or suggest any information regarding treatment of cardiovascular disease.

Even if the Examiner has set forth a *prima facie* case of obviousness, which Applicant vigorously asserts is not the case, such *prima facie* case of obviousness would be successfully rebutted by Applicant's evidence of surprising and unexpected superior results as discussed above.

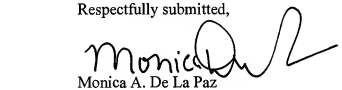
In view of the foregoing, there is no *prima facie* case of obviousness. Therefore, it is respectfully requested that the rejection of claims 15, 17, 19, 38, 24, 54, 56, 58, 60, and 62 as

being unpatentable under 35 U.S.C. §103(a) based on Kornowski and Hu in view of Waller should be withdrawn.

E. Conclusion

In view of the foregoing, it is respectfully submitted that each of the pending claims is in condition for allowance, and a Notice of Allowance is earnestly solicited. The Examiner is invited to contact the undersigned attorney at (512) 536-5639 with any questions, comments or suggestions relating to the referenced patent application.

Respectfully submitted,



Monica A. De La Paz
Reg. No. 54,662
Attorney for Applicants

FULBRIGHT & JAWORSKI L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
512.474.5201 (telephone)
512.536.4598 (fax)

Date: October 31, 2007

EXHIBIT 1

Editorial

Therapeutic Angiogenesis
The New Electrophysiology?

Cam Patterson, MD; Marschall S. Runge, MD, PhD

Approximately 15 million patients suffer from coronary and peripheral atherosclerotic diseases in the United States alone.[1] The evolving development of medical and surgical therapies has significantly improved the physician's ability to manage these patients, yet many continue to suffer debilitating symptoms from their disease and remain at risk for myocardial infarction, limb loss, and death. This clinical imperative, coupled with rapid advances in molecular biology, has led to the exploration of a plethora of therapeutic angiogeuesis strategies. A method described in this issue of *Circulation*[2] offers yet another means by which angiogenesis may be achieved and thus potentially opens a new chapter in the ongoing search for novel approaches for the treatment of ischemia.

See p 2682

Angiogenic and Antiangiogenic Therapy

In placing the novelty of the approach by Kanno et al into perspective, it is worth considering the important advances made during the last decade in the field of therapeutic angiogenesis. The investigators at the forefront of this field have not used a traditional pharmaceutical approach to search for potential angiogenic agents effective against vascular disease. Instead, they have chosen to arm themselves with the fruits of the molecular biology revolution by harnessing the power of endogenous human angiogenic factors. Vascular endothelial growth factor (VEGF) is the most potent and specific endogenous angiogenic factor yet identified, so it makes sense that it would have drawn the attention of cardiologists interested in angiogenic therapies. The demonstration that intra-arterial injection of recombinant VEGF could induce collateral formation in ischemic rabbit hindlimbs[3] suggested that VEGF would be a useful angiogenic agent, and this has subsequently been confirmed by an impressive corpus of preclinical data.

Phase I and II clinical trials are now under way to test the effects of VEGF in patients with ischemic vascular disease, and preliminary results from some of these trials have been published.[4,5] Two methods are being used to deliver VEGF: intra-arterial injection of recombinant VEGF protein, or [illegible]. Preliminary data have been quite promising and promote the general concept that VEGF therapy may be beneficial in humans with vascular disease. However, practical and potential obstacles exist when VEGF is delivered either as a protein or through gene-therapy approaches, which may, to a certain extent, temper the enthusiasm generated by these preliminary studies. Intra-arterial injection of VEGF (for instance, into the coronary circulation) is invasive (and thus costly). Intravascular administration is also sure to result in some systemic delivery of VEGF, which raises at least the theoretical possibility of remote effects such as enhancement of tumor growth, diabetic retinopathy, and other diseases with prominent angiogenic components. Gene-therapy approaches also have the disadvantage of requiring systemic delivery. In addition, the short- and long-term consequences of administering genetic vectors in humans are largely unexplored and need to be addressed before their large-scale use can be recommended.

If only it were so simple. As more has been learned about angiogenesis, it is increasingly apparent that distinct genes regulate different aspects of the process. It has been known for several years that both VEGF and basic fibroblast growth factor are potent angiogenic factors deployed by tumors. This has been part of the rationale for the development of the angiostatins, potent antiangiogenic factors that inhibit tumor-stimulated angiogenesis.[6] However, numerous investigators have now demonstrated that simply initiating (or inhibiting) vascular endothelial cell migration may be insufficient to modulate new vessel formation, and other factors have been identified that are necessary for the complete assembly of new blood vessels. The receptor tyrosine kinases Tie-1 and Tie-2 and their ligands and inhibitors (the angiopoietins) fall into this class.[7] Yet another key component in this molecular cascade are the recently reported Eph receptors, a large family of receptor tyrosine kinases that are distinct from the receptors for VEGF, Tie-1 and Tie-2. The Eph receptors appear to be critical in early determination of whether a developing vessel will become an artery or a vein.[8]

Electrical Stimulation of Angiogenesis

Because of this complexity at the molecular level, the study described by Kanno et al[2] is of particular interest. They demonstrate a novel method whereby VEGF production and angiogenesis can be stimulated: low-intensity (below contraction threshold) electrical stimulation of skeletal muscle. Knowing that stimulated contraction of skeletal muscle induces angiogenesis and VEGF production,[9,10] the authors

The opinions expressed in this editorial are not necessarily those of the editors or of the American Heart Association.

From the Division of Cardiology and Sealy Center for Molecular Cardiology, University of Texas Medical Branch, Galveston, Tex.

Correspondence to Marschall S. Runge, MD, PhD, University of Texas Medical Branch, 9.138 Medical Research Bldg, 301 University Blvd, Galveston, TX 77555-1064. E-mail mrunge@utmb.edu

(*Circulation*. 1999;99:2614-2616.)

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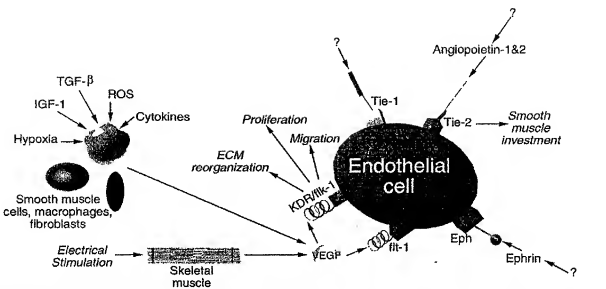

Mechanisms regulating VEGF expression and angiogenesis. IGF-1 indicates insulin-like growth factor-1; TGF-β, transforming growth factor-β; ROS, reactive oxygen species; and ECM, extracellular matrix.

speculated that this effect might be due, at least in part, to electrical stimulation per se rather than to contraction. This is not an unreasonable assumption, because low-intensity electrical stimulation can have significant cellular effects on noncontractile cells.[11]

Some will probably believe such an approach to be hopelessly simple and unlikely to succeed. To be certain, the hypothesis of Kanno et al is far from proven for the reasons discussed below. However, imagine the present status of percutaneous coronary revascularization had Andreas Gruentzig investigated a pharmacological or molecular approach to lessening symptomatic coronary artery stenoses. For this reason alone, it is worth considering the observations of Kanno et al in some detail.

To test their hypothesis, the authors first examined the effect of subcontractile electrical stimulation on skeletal muscle–like cells in culture and found that VEGF production was induced by electrical stimulation in a frequency-dependent manner. Having shown this effect in vitro, the authors used the rabbit ischemic hindlimb model to examine whether low-intensity electrical stimulation might also increase VEGF expression and capillary density in vivo. Electrodes were implanted into ischemic tibialis anterior muscles 1 week after femoral artery ligation, and low-intensity, low-frequency electrical stimulation was applied for another week. The authors detected increased amounts of VEGF protein after this treatment, as measured by immunohistochemistry. This increase in VEGF was accompanied by increases in blood flow and capillary density in the ipsilateral, electrically stimulated limb but not in the contralateral limb, suggesting that angiogenesis was indeed enhanced by this treatment.

In this study, the authors have examined the effect of electrical stimulation on VEGF expression and angiogenesis only in the ischemic muscle bed itself and not in tissues at a distance from the ischemic capillary bed or in nonischemic tissues. This is unfortunate because the goal of angiogenic therapy for ischemic vascular disease might be not only to increase capillary density in ischemic muscle but also to increase collateral formation around stenosed or occluded arteries. Collateral formation in response to ischemia occurs at a distance from the ischemic zone and differs from capillary sprouting histologically, arises more rapidly and by different mechanisms than does capillary sprouting, and is not hypoxia driven.[12] Because induction of collateral formation might be a more efficient goal in angiogenic therapy (by increasing blood supply to an entire target organ rather than to a single ischemic bed), it will be interesting to determine whether electrical stimulation can also induce collateral formation or whether its effects will be limited to induction of capillary sprouting in ischemic tissues.

Because one of the concerns of angiogenic therapies for vascular disease is that systemic effects of these therapies distant from the ischemic zone may adversely affect the patient, it is unfortunate that measurements of VEGF in the venous circulation of treated rabbits were not performed in the present report. This concern is not entirely without basis: plasmid-based delivery of VEGF in humans causes measurable increases in venous VEGF protein levels and has resulted in contralateral limb edema in some patients, indicating that systemic delivery and at least some systemic sequelae do occur.[4] What is the ideal radius for the effects of VEGF? If the effect of electrical stimulation on VEGF expression is strictly local, then there are at least theoretical reasons to believe that its safety profile might be more favorable than angiogenic therapies that result in systemic delivery of VEGF.

A broader, unresolved issue raised by the present study is whether differences exist when angiogenesis is elicited by exogenously administered growth factors rather than by

therapies designed to stimulate endogenous angiogenic pathways. It must be recognized that at this point in time, the favorable results from human angiogenic gene-therapy trials have been disclosed primarily as series of case reports. Furthermore, whereas most of the animal studies using VEGF in the setting of ischemic vascular disease have demonstrated favorable effects, some have demonstrated no effects of VEGF on parameters such as reendothelialization.[13] In addition, some data suggest that VEGF may play a role in the microvascular complications of diabetes,[14] and VEGF was shown to worsen neointimal lesion size without affecting collateral formation in a canine model of ischemic coronary disease.[15] A better understanding of endogenous pathways regulating VEGF activity may help to explain why VEGF may exert both salutary and deleterious vascular effects, which ultimately could lead to more efficacious angiogenic therapies.

Moreover, the potential importance of the other molecular guardians of angiogenesis described above remains largely untested in animal models or humans. Clearly, the potential therapeutic impact of these additional pathways is not addressed in ongoing VEGF-based clinical studies. Because it is unknown whether these pathways are also affected by electrical stimulation, addressing this issue may provide intriguing insight into the most effective approaches toward achieving therapeutic angiogenesis. To revisit the angioplasty paradigm, a multitude of pathways are activated by balloon injury, and only in the past few years have the molecular puzzles begun to be solved.

Modern electrical-stimulation therapies are used to treat chronic pain syndromes and may facilitate the healing of fractures and soft-tissue wounds.[16] On the basis of the present report, it is tempting to speculate that induction of VEGF may contribute to the salutary effects of electrical stimulation in these settings. Regardless of whether this is the case, there is considerable accumulated experience in the use of electrical stimulation in the clinical arena. It can be applied transcutaneously and is thus remarkably safe, relatively inexpensive, and easy to administer. In contrast with intravascular injection of VEGF, induction of endogenous VEGF by electrical stimulation could be performed noninvasively and repeatedly. Unlike gene therapy, electrical stimulation has been used successfully for decades, and untoward effects are unlikely if this technique is used to stimulate VEGF expression for the treatment of ischemic vascular disease.

Although angiogenic therapy holds promise for the treatment of ischemic vascular disease, we are still in the earliest stages of testing its effectiveness and of determining the best way to enhance collateral formation and new capillary growth in ischemic tissues. It will not be surprising if molecular and nonmolecular strategies become alternative or even complementary therapies to induce angiogenesis. Indeed, electrical stimulation may be yet another method for augmenting angiogenesis, and studies to examine its effectiveness in humans with ischemic vascular disease will be eagerly awaited.

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KEY WORDS: Editorials ■ growth substances ■ angiogenesis ■ atherosclerosis

EXHIBIT 2

Brant

CARDIOPULMONARY SUPPORT AND PHYSIOLOGY

BIOLOGIC BYPASS WITH THE USE OF ADENOVIRUS-MEDIATED GENE TRANSFER OF THE COMPLEMENTARY DEOXYRIBONUCLEIC ACID FOR VASCULAR ENDOTHELIAL GROWTH FACTOR 121 IMPROVES MYOCARDIAL PERFUSION AND FUNCTION IN THE ISCHEMIC PORCINE HEART

Charles A. Mack, MD[a,b]
Shailen R. Patel, MD[a,b]
Eric A. Schwarz, MD[a,b]
Pat Zanzonico, PhD[c]
Rebecca T. Hahn, MD[d]
Arzu Ilercil, MD[d]
Richard B. Devereux, MD[d]
Stanley J. Goldsmith, MD[c]
Timothy F. Christian, MD[d]
Timothy A. Sanborn, MD[d]
Imre Kovesdi, PhD[e]
Neil Hackett, PhD[b]
O. Wayne Isom, MD[a]
Ronald G. Crystal, MD[b*]
Todd K. Rosengart, MD[a*]

Objectives: Vascular endothelial growth factor (VEGF), a potent angiogenic mediator, can be delivered to targeted tissues by means of a replication-deficient adenovirus (Ad) vector. We hypothesized that direct administration of an Ad vector expressing the VEGF$_{121}$ complementary deoxyribonucleic acid ($Ad_{CV}VEGF121.10$) into regions of ischemic myocardium would enhance collateral vessel formation and improve regional perfusion and function. *Methods:* Yorkshire swine underwent thoracotomy and placement of an Ameroid constrictor (Research Instruments & MFG, Corvallis, Ore.) on the circumflex coronary artery. Three weeks later, myocardial perfusion and function were assessed by single photon emission computed tomography imaging (SPECT) with ^{99m}Tc-labeled sestamibi and by echocardiography during rest and stress. $Ad_{CV}VEGF121.10$ ($n = 7$) or the control vector, AdNull ($n = 8$), was administered directly into the myocardium at 10 sites in the circumflex distribution (10^8 pfu/site). Four weeks later, these studies were repeated and ex vivo angiography was performed. *Results:* SPECT imaging 4 weeks after vector administration demonstrated significant reduction in the ischemic area at stress in $Ad_{CV}VEGF121.10$-treated animals compared with AdNull control animals ($p = 0.005$). Stress echocardiography at the same time demonstrated improved segmental wall thickening in $Ad_{CV}VEGF121.10$ animals compared with AdNull control animals ($p = 0.03$), with $Ad_{CV}VEGF121.10$ animals showing nearly normalized function in the circumflex distribution. Collateral vessel development assessed by angiography was also significantly greater in $Ad_{CV}VEGF121.10$ animals than in AdNull control animals ($p = 0.04$), with almost complete reconstitution of circumflex filling in $Ad_{CV}VEGF121.10$ animals. *Conclusions:* An Ad vector expressing the VEGF$_{121}$ cDNA induces collateral vessel development in ischemic myocardium and results in significant improvement in both myocardial perfusion and function. Such a strategy may be useful in patients with ischemic heart disease in whom complete revascularization is not possible. (J Thorac Cardiovasc Surg 1998;115:168-77)

From the Department of Cardiothoracic Surgery,[a] Division of Pulmonary and Critical Care Medicine,[b] Division of Nuclear Medicine,[c] and Division of Cardiology,[d] The New York Hospital–Cornell Medical Center, New York, N.Y., and GenVec, Inc.,[e] Rockville, Md.

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Address for reprints: The New York Hospital–Cornell Medical Center, New York, NY 10021.

*R.G.C. and T.K.R. contributed equally as senior investigators in this study.

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Despite continued advances in the treatment of ischemic heart disease, a large population of individuals with diffuse coronary artery disease exists for whom conventional therapies such as percutaneous angioplasty and bypass surgery provide little or no benefit. A new strategy that may be applicable to such patients is "therapeutic angiogenesis," the induction of new blood vessel development into areas with limited blood flow.[1, 2] Therapeutic angiogenesis capitalizes on the discovery of protein mediators that elicit angiogenesis, a complex process that includes the migration and proliferation of endothelial cells, vascular tube formation, and linkage to the preexisting vascular network.[3, 4] Several of these protein mediators have been shown to promote revascularization of ischemic tissues in models of myocardial or peripheral ischemia.[1-3, 5-9]

Vascular endothelial growth factor (VEGF), a homodimeric 34 to 46 kDa heparin-binding glycoprotein, is the most specific of the known angiogenic mediators because of localization of its receptors almost exclusively on endothelial cells.[10-12] The human VEGF gene is expressed as four isoforms secondary to posttranscriptional splicing, producing proteins of 121, 165, 189, and 206 residues. The observations that VEGF and VEGF receptors (flk-1/KDR and flt-1) are up-regulated under ischemic conditions is consistent with the concept that VEGF is an endogenous mediator of angiogenesis.[11-13]

Gene therapy, in which the complementary deoxyribonucleic acid (cDNA) for VEGF is delivered directly to tissues, has the potential to induce localized VEGF expression that is of limited duration, thus avoiding the toxicity and promiscuous angiogenesis potentially associated with systemic protein therapy.[14-18] In examining this hypothesis, the present study uses a porcine myocardial ischemia model to demonstrate that direct administration of a replication-deficient adenovirus (Ad) vector coding for the human $VEGF_{121}$ cDNA (Ad_{GV}-VEGF121.10) into ischemic myocardium induces a "biologic bypass"—collateralization around a site of coronary occlusion, with concomitant improvement in regional myocardial perfusion and function during stress-induced myocardial ischemia.

Methods

Experimental model of myocardial ischemia. A model of chronic myocardial ischemia was created in Yorkshire swine (28 to 30 kg). All animal care procedures were in accordance with institutional guidelines. Animals were sedated with intramuscular tiletamine and zolazepam (Telazol, 3.3 mg/kg) and xylazine (0.10 mg/kg), intubated, and sedation was maintained with 0.5% to 2.0% isoflurane. A limited left thoracotomy was performed in a sterile fashion through the fifth intercostal space and a small incision was made in the pericardium. A 2.5 mm internal diameter Ameroid constrictor (Research Instruments & MFG, Corvallis, Ore.) was placed around the circumflex artery as proximally as possible. Topical lidocaine 1% solution was applied to the circumflex artery at the Ameroid constrictor site to prevent coronary artery spasm. The pericardium and chest were then closed and the animal was allowed to recover.

Ad vectors. The replication-deficient vector Ad_{GV}-VEGF121.10 is an E1a$^-$, partial E1b$^-$, partial E3$^-$Ad vector that contains an expression cassette in the E1 position (right to left) containing the cytomegalovirus immediate early promoter/enhancer, an artificial splice sequence, the human $VEGF_{121}$ cDNA, and the SV40 polyA/stop signal. AdNull (similar to Ad_{GV}VEGF121.10, but with no gene in the expression cassette) was used as a control vector.[19] All Ad vectors were propagated and titrated in 293 cells, purified by cesium chloride density purification, dialyzed, and stored at −70° C.[20] The viral stocks were demonstrated to be free of replication-competent wild type Ad. Biologic activity of the $VEGF_{121}$ transgene product was confirmed by demonstrating proliferation of human umbilical vein endothelial cells using [^{3}H]thymidine incorporation, and in vivo confirmation of transgene expression was determined by enzyme-linked immunosorbent assay analysis of myocardial biopsy specimens obtained from AdVEGF121.10 injection sites 3 days after vector administration.[17]

In vivo administration of Ad vectors. Three weeks after Ameroid constrictor placement, the left thoracotomy was reopened and administration of the therapeutic vector, Ad_{GV}VEGF121.10, or the control vector, AdNull, was performed by direct myocardial injection (Fig. 1). Each vector was injected at 10 sites, each in 100 μl phosphate-buffered saline solution, pH 7.4, in the circumflex distribution (10^8 pfu/injection). Pacing wires were placed in the left atrial appendage and tunneled subcutaneously for subsequent stress technetium 99m (^{99m}Tc)-labeled sestamibi (Cardiolite, DuPont Pharma, N. Billerica, Mass.) assessment of regional myocardial perfusion by single photon emission computed tomography (SPECT) and echocardiographic assessment of regional wall thickening.

^{99m}Tc-labeled sestamibi assessment of regional myocardial perfusion. Regional myocardial perfusion was evaluated during rest and stress 3 weeks and 7 weeks after placement of the Ameroid constrictor by means ^{99m}Tc-sestamibi SPECT. During rapid atrial pacing at 200 beats/min, animals received intravenous injections of a 5 mCi bolus of ^{99m}Tc-sestamibi and pacing was continued for approximately 3 minutes. The animals were then placed in the prone position in an ADAC Vertex dual detector gamma camera system (ADAC Laboratories, Milpitas, Calif.). A nongated SPECT study was then acquired in a "step-and-shoot" mode over a 180-degree body-contouring orbit. The animal was allowed to return to baseline heart rate and was then received an injection of a 25 mCi bolus of ^{99m}Tc-sestamibi before obtaining a rest SPECT, acquired in an analogous fashion.

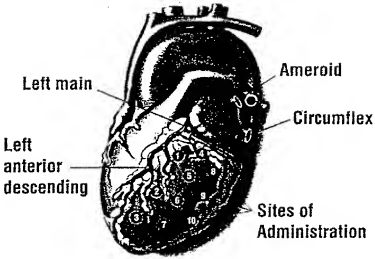

Fig. 1. Schema of the experimental design. Three weeks after Ameroid constrictor placement, animals were administered either $Ad_{GV}VEGF121.10$ ($n = 7$) or the control vector AdNull ($n = 8$) at 10 sites (10^8 pfu/site) distributed throughout the left ventricular region between the circumflex and left anterior descending coronary arteries, as indicated.

The rest and stress SPECT studies were processed in a blinded fashion with the use of an integrated ADAC Pegasys computer. Stress and rest circumferential count profiles (polar plots) at the midventricular level were constructed by dividing the midventricular short-axis image into 60 angular segments centered on the ventricular cavity, determining the number of counts per segment, normalizing the number of counts in each segment to the segment with the maximum number of counts (assigned a reference value of 100), and plotting the normalized counts per segment versus the angular position of the segment. The polar plots were transferred to ASCII files for further analysis with the program SIGMAPLOT (Jandel Scientific, Corte Madera, Calif.).

For each animal, the extent of myocardial ischemia ("area") was determined from the difference between the rest and stress polar plots. The maximum severity of ischemia ("ischemia maximum") in the circumflex distribution was determined by ascertaining the point of greatest difference between the rest and stress plots and measuring the difference in the plots at that point. The percent improvement in myocardial perfusion for each animal was calculated for these two parameters as ("parameter" at 3 weeks − "parameter" at 7 weeks × 100)/("parameter" at 3 weeks).

Echocardiographic assessment of regional myocardial function. Baseline regional myocardial function was assessed by echocardiography at rest and during stress at the time of vector administration. Animals were sedated and placed in the left lateral decubitus position, and standard two-dimensional and M-mode transthoracic images were obtained with an HP2500 echocardiographic machine and a 3.0/3.5 MHz dual-frequency transthoracic transducer (Hewlett-Packard, Andover, Mass.). From the right parasternal approach, short-axis, midpapillary views were obtained at rest for 3 minutes. The animals then underwent rapid left atrial pacing in a stepwise fashion to the target ventricular rate of 200 beats/min, at which time imaging was recorded for an additional 3 minutes.

Regional wall thickening was determined by a single experienced investigator in a blinded fashion, tracing the endocardial and epicardial surfaces of the left ventricle in both diastole and systole using a Digisonics CardioRevue System (Digisonics Inc., Houston, Tex.). Systolic wall thickening in each of six equal radial 60-degree segments was defined as mean systolic wall thickness − mean diastolic wall thickness. Fractional wall thickening was calculated as mean systolic wall thickening/mean diastolic wall thickness. The ischemic and nonischemic zones for each animal were defined from rapid atrial pacing images at 3 weeks (baseline ischemia) as the two contiguous segments with the lowest and highest fractional wall thickening, respectively. This corresponded in all cases with the circumflex region and the septum, respectively. The same zones for each animal were analyzed in rapid atrial pacing images at 7 weeks.

Ex vivo coronary angiography. When each animal was put to death (4 weeks after vector administration), the heart was arrested with 40 mEq of KCl and then perfusion-fixed at 100 mm Hg with 1 L of McDowell-Trump fixative (4% formaldehyde, 1% glutaraldehyde, 1% NaH_2PO_4 and 0.3% NaOH adjusted to pH 7.2). Ex vivo coronary angiography was performed by the same angiographer in a blinded fashion using a 5F end-hole wedge balloon catheter (Arrow Inc., Reading, Pa.) placed in the left main coronary artery. By means of cinefluoroscopy in

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Table I. *Quantitative assessment of regional stress-induced myocardial ischemia by ^{99m}Tc-labeled sestamibi SPECT imaging*

	*Ischemic area**			*Ischemic maximum**		
Time of study	*Ad_{GV}VEGF121 (n = 8)*	*AdNull (n = 7)*	*p Value*	*Ad_{GV}VEGF121 (n = 8)*	*AdNull (n = 7)*	*p Value*
3 wk (vector administered)	3570 ± 640	3200 ± 390	0.8	30.7 ± 3.6	26.8 ± 2.1	0.2
7 wk	750 ± 220	2140 ± 400	0.005	11.0 ± 2.2	22.3 ± 1.7	0.04

*Values are mean ± standard error of the mean; regional stress-induced was quantified by determining the area of ischemia and ischemia maximum as described in Fig. 2, *A* and the *Methods* section.

the standard right anterior oblique projection with continuous image acquisition, 5 ml of contrast medium (Hypaque-76, Nycomed Inc., New York, N.Y.) was injected at a continuous rate until the entire left anterior descending coronary artery and its branches were completely opacified.[21] Collateral vessels from the left anterior descending coronary artery, which reconstituted the circumflex coronary artery or obtuse marginal branch of the circumflex coronary artery, were quantified by three blinded observers using the grading method of Rentrop and associates[22] as follows: 0 = no filling of collateral vessels; 1 = filling of collateral branches of the circumflex or obtuse marginal branch without visualization of the epicardial segment; 2 and 3 = partial or complete filling of the epicardial segment of the circumflex or obtuse marginal artery via collateral vessels, respectively.

Histologic evaluation. After angiography, the left ventricle of each heart was sectioned into three rings in the short axis. Forty 5 μm histologic sections from each heart were taken at equidistant intervals around the basal and midventricular rings, processed through paraffin, and stained with hematoxylin and eosin. Histologic evidence of infarction and inflammation for each tissue section was graded by a pathologist blinded to treatment on a scale of 0 to 4 as follows: 0 = none; 1 = one to three small areas involved; 2 = less than 10% section surface; 3 = more than 10% up to 50% section surface; and 4 = more than 50% section surface.

Statistical analysis. Treatment was assigned in an alternating consecutive fashion to a total enrollment of 15 animals that could be evaluated for efficacy, with myocardial ischemia area defined as the primary outcome variable. Statistical analysis was carried out by means of the Mann-Whitney nonparametric test. All results are expressed as mean ± standard error of the mean.

Results

Overall assessment. All of the 19 animals entered into the study (Ad_{GV}VEGF121.10, n = 9; AdNull, n = 10) survived until put to death 7 weeks after placement of the Ameroid constrictor, without clinical evidence of toxicity. At 3 weeks (i.e., before therapy), four of the 19 pigs (Ad_{GV}VEGF121.10, n = 2; AdNull treated, n = 2) had evidence of myocardial infarction in the circumflex region, as demonstrated by (1) a fixed defect (no difference between rest and stress) in the circumflex zone of the ^{99m}Tc-sestamibi SPECT images and (2) a thinned, akinetic posterolateral region of the left ventricle in short-axis views during echocardiography at rest. Consistent with the ^{99m}Tc-sestamibi SPECT and echocardiography suggesting myocardial infarction 3 weeks after Ameroid constrictor placement, the gross pathologic evaluation 4 weeks later showed myocardial scarring and thinning of at least 25% of the total ventricular mass. All four pigs in this subgroup also had histologic evidence of large transmural infarction. On the basis of these data, these four animals were excluded from further analysis. Thus the group of animals evaluated for efficacy of therapy included seven Ad_{GV}-VEGF121.10-treated animals and eight AdNull (control) animals.

Table II. *Stress-induced regional contractile dysfunction assessed by two-dimensional echocardiography*

	*Difference in fractional wall thickening (nonischemic zone − ischemic zone, %)**		
Time of study	*Ad_{GV}VEGF121 (n = 8)*	*AdNull (n = 7)*	*p Value*
3 wk (vector administered)	16 ± 4.2	17.2 ± 4.4	0.9
7 wk	−0.06 ± 3.3	12.4 ± 4.0	0.03

Values are mean ± standard error of the mean; Short-axis images of two-dimensional echocardiograms were divided into six radial segments, and the fractional wall thickening (systolic wall thickening as a percentage of diastolic wall thickness) of the ischemic zones was assessed as described in the *Methods* section. The percent difference in fractional wall thickening of the nonischemic zone minus the ischemic zone was calculated for AdNull and Ad_{GV}VEGF121.10; zero difference in fractional wall thickening signifies equivalent function in the ischemic and nonischemic zones.

In vivo expression of the Ad_{GV}VEGF.10 vector was confirmed by demonstrating local myocardial VEGF expression after myocardial injection of 10^8 pfu of Ad_{GV}VEGF121.10 (n = 3). Three days after administration of the vector, myocardial levels were 0.75 ± 0.25 ng/mg protein, compared with back-

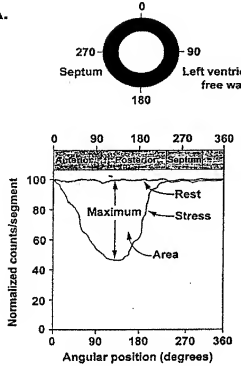

Fig. 2. Quantitative assessment of regional stress-induced myocardial ischemia with ^{99m}Tc-labeled sestamibi SPECT imaging. A, Schema of method using circumferential count profiles. Short-axis ^{99m}Tc-sestamibi images at the midventricular level were analyzed as described in the *Methods* section. The extent and severity (area) of myocardial ischemia was determined from the difference between the rest and stress circumferential count profile curves. The greatest severity of ischemia (ischemia maximum) in the circumflex distribution was determined as the greatest difference between the rest and stress circumferential count profiles.

ground expression in AdNull-treated animals ($p = 0.01$).

Ad_{GV}VEGF121.10-mediated increase in myocardial perfusion. Circumferential count profiles (polar plots) of ^{99m}Tc-sestamibi SPECT data from the midventricular level were used to quantify (1) the extent and severity of ischemia ("area") and (2) the most severe ischemia ("ischemia maximum") (Fig. 2, *A*). Circumferential plots of rest images obtained at 3 weeks typically demonstrated minimal perfusion defects, compared with plots of stress (pacing) images, which revealed decreased perfusion in the posterolateral region, corresponding to the occluded circumflex coronary artery distribution (Fig. 2, *B* and *D*). The ischemic area and ischemia maximum were characteristically unchanged from baseline in AdNull animals assessed 4 weeks after vector administration (Fig. 2, *B* and *C*). In contrast, Ad_{GV}VEGF121.10 animals demonstrated improvement in myocardial perfusion 4 weeks after vector administration, as demonstrated by decreases in the ischemic area and ischemic maximum compared with baseline (Fig. 2, *D* and *E*). Corresponding changes were noted at the apical, midventricular, and basal levels.

The ischemic area was similar in both the Ad_{GV}VEGF121.10 and AdNull control animals at the time of vector administration (Table I). In contrast, the ischemic area was significantly reduced at 7 weeks in the Ad_{GV}VEGF121.10 animals compared with the AdNull animals. The "percent improvement" in the area of ischemia of each animal 4 weeks after vector administration, compared with baseline, was approximately 2.4-fold greater in the Ad_{GV}VEGF121.10 animals than in the AdNull animals (75% ± 6% vs 32% ± 11%, respectively, $p = 0.01$).

The ischemia maximum in the circumflex distribution was also the same for the Ad_{GV}VEGF121.10 animals and AdNull control animals at 3 weeks (Table I). In contrast, 4 weeks after vector administration, the ischemia maximum was significantly decreased in the Ad_{GV}VEGF121.10 animals than in the AdNull control animals. Similarly, the "percent improvement" in the ischemia maximum was 2.5-fold greater in Ad_{GV}VEGF121.10 animals than in the AdNull control animals (56% ± 8% vs 22% ± 6%, $p = 0.01$).

Ad_{GV}VEGF121.10-mediated improvement in myocardial function. Three weeks after Ameroid constrictor placement, myocardial function in the ischemic circumflex region compared with the nonischemic septum was similar in the Ad_{GV}VEGF121.10 group compared with AdNull controls as assessed by fractional wall thickening during rapid atrial pacing (Table II). In contrast, by 4 weeks after vector administration, Ad_{GV}VEGF121.10-treated animals demonstrated significantly greater improvement in fractional wall thickening during rapid atrial pacing than did AdNull control animals. Strikingly, contractile function in the circumflex segment of the Ad_{GV}VEGF121.10 group approximated that of the septal (control) segment, as reflected by an ischemic minus nonischemic zone difference of "zero" in this analysis.

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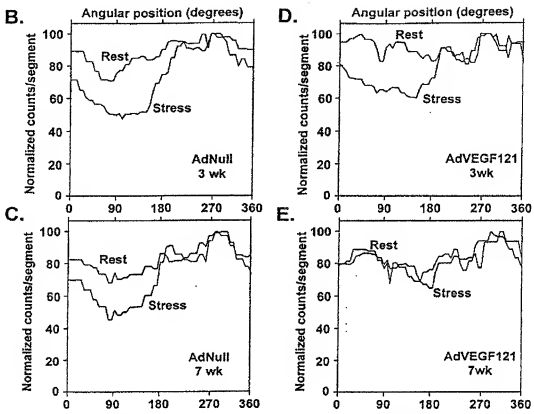

Fig. 2. Cont'd. B, Representative circumferential count profiles of an AdNull-treated animal at 3 weeks (at the time of vector administration) at rest *(upper part of the panel)* and stress (atrial pacing; *at the lower part of the panel),* showing a perfusion defect in the posterolateral region. C, Same animal as in panel B, but at 7 weeks. There is minimal decrease in area of ischemia and ischemia maximum compared with 3 weeks. D, Representative circumferential count profiles of an Ad_{GV}VEGF121-treated animal at 3 weeks (at the time of vector administration) at rest and stress (atrial pacing), showing a perfusion defect in the posterolateral region. E, Same animal as in panel D, but at 7 weeks; there is a marked decrease in both area of ischemia and ischemia maximum compared with observations at 3 weeks.

Angiographic assessment of coronary vessels. Ex vivo angiography performed 4 weeks after vector administration confirmed complete occlusion of the proximal circumflex coronary artery by the Ameroid constrictor in all animals. AdNull-treated animals characteristically demonstrated only partial filling of the obtuse marginal and circumflex coronary arteries (Fig. 3, *A*). In contrast, animals that received Ad_{GV}VEGF121.10 typically demonstrated nearly complete reconstitution of both the obtuse marginal and circumflex coronary circulations (Fig. 3, *B* and *C*).

The collateral grade for the obtuse marginal and circumflex coronary arteries was significantly greater in the Ad_{GV}VEGF121.10 animals than in the AdNull animals (Table III). Finally, the total number of angiographically visible collateral vessels filling the circumflex and obtuse marginal arteries was significantly greater in the Ad_{GV}VEGF121.10 animals than in the AdNull animals (Table III).

Histologic assessment. The myocardium in 13 of the 15 animals in the study was available for assessment of inflammation (Ad_{GV}VEGF121.10, $n = 5$;

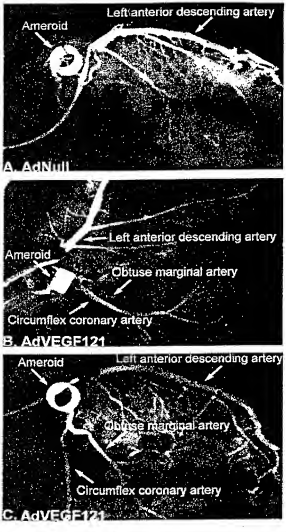

Fig. 3. Representative ex vivo angiograms of pig hearts 7 weeks after placement of Ameroid constrictors. The vectors were administered 3 weeks after placement of the Ameroid constrictor. **A**, AdNull. **B** and **C**, Ad_{CV}-VEGF121.10 (referred to as AdVEGF121). The Ameroid constrictor completely occludes the circumflex artery in both the AdNull and AdVEGF121-treated animals. The AdNull-treated animal demonstrates only minimal filling of the distal circumflex artery. In contrast, the AdVEGF121-treated animals show nearly complete reconstitution of the circumflex artery.

AdNull, $n = 8$). Minimal inflammation was detected in the myocardium of these animals evaluated 4 weeks after therapy, with no difference in the extent of inflammation between the Ad_{CV}VEGF121.10 and AdNull groups (overall intensity score 0.3 ± 0.06 *vs* 0.4 ± 0.08, $p = 0.4$).

Table III. *Quantitative assessment of collateral vessel grade and number as assessed by ex vivo angiography*

	Ad_{CV}*VEGF121** ($n = 8$)	*AdNull** ($n = 7$)	*p Value*
Collateral grade (scale: 0-3)			
To obtuse marginal	2.9 ± 0.1	1.8 ± 0.2	0.03
To circumflex	2.8 ± 0.1	1.9 ± 0.3	0.04
Collateral vessel number†	4.0 ± 0.4	2.0 ± 0.3	0.001

*Values are mean ± standard error of the mean determinations of three blinded observers as described in the *Methods* section.
†Number of angiography-visible collateral vessels observed between the left anterior descending and the circumflex or obtuse marginal arteries.

Discussion

This study demonstrates that Ad-mediated transfer of the cDNA of human VEGF isoform 121 directly into the myocardium of Yorkshire swine with an occluded circumflex coronary artery results in significant and physiologically relevant improvement in regional myocardial perfusion and contractile function during stress-induced myocardial ischemia. Importantly, this improvement was associated with increased myocardial collateral vessel development, "biologically bypassing" the experimentally occluded coronary artery segment.

VEGF as a mediator of therapeutic angiogenesis. Considerable information is available suggesting that the VEGF protein can induce angiogenesis in a variety of animal models of ischemia.[2-9, 13] In regard to cardiac ischemia, a single intracoronary bolus of $VEGF_{165}$ improves blood flow to the ischemic region,[9] and continuous infusion of the $VEGF_{165}$ protein via indwelling catheters increases collateral blood flow to ischemic myocardium and the numeric density of intramyocardial distribution vessels.[5] Finally, continuous administration of $VEGF_{165}$ to the surface of the myocardium over 6 weeks reduces the ischemic zone and improves ejection fraction and regional myocardial wall thickening as assessed by magnetic resonance imaging.[8]

Our choice of VEGF for this study is based on these findings and the endothelial cell specificity of VEGF, which offers potential advantages over the use of other growth factors with angiogenic properties (such as the fibroblast growth factors), which are also mitogenic for cells such as fibroblasts and smooth muscle cells, and thus theoretically impose the risk of unwanted fibrosis or

smooth muscle cell hyperplasia.[3, 23, 24] Although the major VEGF isoforms appear to be equipotent in angiogenic potential, our choice of the cDNA coding for the 121 amino acid isoform of VEGF was based on the knowledge that the $VEGF_{121}$ isoform does not bind heparin and thus may diffuse throughout the myocardium more readily than the other isoforms.[11, 12]

Ad-mediated gene transfer as a strategy for VEGF-related therapeutic angiogenesis. Gene therapy holds several potential advantages over a protein-based strategy for therapeutic angiogenesis. Gene transfer provides the equivalent of a "slow-release depot," providing high concentrations of the therapeutic protein for a relatively extended period. Ad vector expressing VEGF provides myocardial expression of VEGF protein for up to 7 days.[17] In contrast, the VEGF protein has a very short biologic half-life in the circulation,[6] and most previous studies have consequently required continuous infusions or repetitive dosing of the growth factor to achieve a therapeutic effect.[5, 8, 15] Another potential advantage of gene therapy over protein-mediated angiogenic therapy is that gene transfer can be strategized to provide delivery of a high concentration of VEGF localized to the ischemic sites. In contrast, systemic administration of a single large bolus of VEGF protein can cause hypotension, and systemic therapy carries the theoretic risk of inducing inappropriate angiogenesis at sites of vascular derangement, or at sites where angiogenesis might have major adverse consequences, such as the retina, the synovium, and in occult tumors.[3, 9, 11, 12, 16]

Although three gene transfer systems—naked plasmids, herpes simplex virus, and Ad—have been used to induce angiogenesis with VEGF in experimental models, we have focused on Ad vectors because their inherent properties make them ideal for use in therapeutic angiogenesis. Ad vectors achieve gene transfer in both dividing and nondividing cells, with high levels of protein expression in cardiovascular relevant sites, such as myocardium, vascular endothelium, and skeletal muscle.[14, 17, 25, 26] The new gene transferred by an Ad vector functions in an epi-chromosomal position and thus carries little risk of inappropriately inserting the newly transferred gene in a critical site of the genome.[27] Most important, whereas long-term expression of angiogenic proteins might induce excessive, disorganized blood vessel formation, Ad vectors are highly efficient at achieving high levels of gene expression in cardiovascular tissues for only 1 to 2 weeks, thus limiting expression to that necessary to induce angiogenesis.[14, 17, 18] Consistent with the concept that Ad vectors can be used to deliver genes relevant to inducing myocardial angiogenesis, an Ad vector has been used to deliver the fibroblast growth factor–5 cDNA to the ischemic porcine myocardium, with resulting increases in perfusion, function, and histologic evidence of angiogenesis.[14]

Host immune response is a potential disadvantage to the use of Ad vectors in some gene transfer applications. With the use of clinical grade vectors, as in the present study, however, inflammation does not appear to be a significant problem. The cellular immune response that likely limits vector expression is actually an advantage for the application of therapeutic angiogenesis, as previously discussed. Finally, although it is unlikely that repeated administrations will be needed, the theoretic development of neutralizing antibodies directed against the Ad vector that might limit the effectiveness of repeated vector administrations can likely be overcome by the use of Ad vectors of different serotypes or by immunosuppressive therapy.[28]

Ad-mediated angiogenesis as a biologic revascularization strategy. The primary significance of the present results is that a one-time direct myocardial administration of an Ad vector containing the $VEGF_{121}$ cDNA induces the development of collateral vessels adequate to enhance myocardial perfusion and ameliorate regional myocardial contractile dysfunction in the setting of stress-induced myocardial ischemia. The observation in control animals of some collateral vessel development is consistent with evidence of the limited, incomplete nature of the endogenous processes of angiogenesis and collateralization that are known to occur in the setting of ischemia. In this context, the therapeutic angiogenesis described in this study can be considered as a logical enhancement of the normal, endogenous response to ischemia. With the evidence that Ad vectors are capable of delivering new genetic information safely to human beings in vivo,[27] the clinical applications of this strategy include its use as a novel therapeutic "biologic bypass" in patients with coronary disease.

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Discussion

Dr. Andrew S. Wechsler *(Richmond, Va.).* This work demonstrates the efficacy of Ad-based gene transfer as a means to achieve a potentially clinically applicable treatment strategy. It far transcends studies that have focused solely on demonstrating the ability to transfer genetic material to the myocardium. Locally enhanced expression of VEGF in the vicinity of ischemic myocardium improved collateral vessel density, regional blood flow, and myocardial function under stress conditions. The induction of new vessel growth by VEGF or its genetic progenitors is so promising that the Food and Drug Administration has recently approved a clinical trial proposed by Isner and his colleagues at the New England Medical Center. In their study, naked DNA coding for VEGF is to be infused into ischemic extremities unsuitable for surgical revascularization.

I have several questions for the authors:

Why did you select the 121 VEGF?

You noted minimal inflammation. Other investigators have consistently observed worrisome degrees of inflammation associated with Ad gene transfer. Why do you think inflammation was so minimal?

Why did you choose an Ad vector over naked DNA or plasmid-based gene transfer?

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Have you performed experiments to determine the duration of gene expression or demonstrated VEGF within the myocardium?

Do you believe you have accelerated time-dependent collateral development or that you have induced a qualitative generation of neocollaterals?

Finally, do you think intravascular administration via the left anterior descending coronary artery would have had a comparable effect, inasmuch as this would allow a more convenient use of this technique if it were to become clinically applicable?

Dr. Mack. We chose the 121 isoform as opposed to the other isoforms because the 121 isoform is known to bind to just one of the VEGF receptors, unlike the other isoforms. Certain inflammatory cells such as monocytes and some carcinoma cell lines are known to express both or one of the VEGF receptors. We thought that the $VEGF_{121}$ isoform would provide us with the most specificity for endothelial cells in that it binds to the Flk-1 receptor and not to the Flt-1 receptor.

In addition, $VEGF_{121}$ does not have as much affinity for heparin as does $VEGF_{165}$ and would diffuse more into the ischemic milieu and therefore potentially enhance any therapeutic effect.

Concerning inflammation, contamination by replication-competent viral particles to a large extent determines the degree of inflammation when Ad vectors are used. The vector that we used was free of replication-competent particles up to 10^8 pfu. As Ad vector technology advances, the degree of inflammation potentially can become less and less.

Disadvantages of the Ad vector for many of the inherited diseases, in that it expresses the transgene for only a limited duration, is one of the main reasons why we chose an Ad for this application. It was our hypothesis that a localized, yet transient degree of expression would be just enough to induce a neovascular response. Any prolonged degree of expression raises the concern of disorganized angiogenesis, inappropriate angiogenesis at other sites, such as the retina, occult tumors, and arthritic joints. The fact that the first-generation Ad vectors are known to express the transgene only transiently is a true advantage for this application.

Regarding the duration of expression, in this porcine model we have done only very preliminary studies looking at gene expression and quantifying gene expression. Prior work in our laboratory done by Christopher Magovern has demonstrated that an Ad vector expressing $VEGF_{165}$, expression can be as long as 7 to 14 days. Although we have not studied the duration of expression to date in the porcine model, we are planning to do that study and validate the fact that expression is transient.

Dr. Wechsler inquired about the mechanism contributing to our results, whether it is an increase in the endogenous response or a new generation of blood vessels. It is probably a combination of several things. The endogenous response probably is enhanced to some degree; however, other investigators, using VEGF and other growth factors and certain mitotic assays like bromodeoxyuridine and proliferating cell nuclear antigen, have demonstrated that a new generation of blood vessels develops. Therefore the collateral vessel response contributing to improved blood flow is likely a multifactorial process.

As far as the route of administration through the left anterior descending coronary artery, it was our purpose to directly inject the vector into the myocardium to avoid any systemic toxicity. Catheter-related devices that allow injection into the coronary vessel raise the concern of a systemic administration.

Dr. Margaret D. Allen *(Seattle, Wash.).* Did antibodies to Ad develop?

Dr. Mack. We looked at neutralizing antibodies at the time of Ameroid constrictor placement, the time of vector administration, and when the animals were put to death. Neutralizing antibodies to the Ad were observed at the time of sacrifice at a very low level. This has to be taken in context with what has been published for the most part in small animals, where the same dose of vector is given in an animal that is perhaps a thousand times smaller. The degree of neutralization in these experiments was relatively minimal.

Dr. Allen. That would be interesting, because it might affect retreatment in the future, for instance, for an ischemic patient who might need more than one treatment. It would be interesting to know whether the intramyocardial injection perhaps is less likely to induce an antibody response than an intravenous injection.

Dr. Mack. That is a very insightful point. Perhaps the route of administration does play a role here. As far as being able to administer the vector twice, there are 49 different Ad serotypes, and studies have been published whereby just altering the serotype can circumvent neutralizing immunity. This does not escape the cytotoxic T-cell response that clears the virus. However, clearly a repeat administration using an alternate serotype or immunomodulation can be achieved.

Mr. Reida M. El Oakley *(London, England).* I agree with Dr. Wechsler that your data would be more informative if you included two more control groups, one with the plasmid only and one with saline solution or nothing altogether.

Dr. Mack. We actually have in progress a treatment group in which the animals receive no injection at the second operation. Preliminarily, there appears to be no difference between that group and our AdNull-treated controls.